

Title	Humoral Cytotoxic Antibodies in Urogenital Carcinoma Patients
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Citation	泌尿器科紀要 (1977), 23(3): 207-212
Issue Date	1977-04
URL	http://hdl.handle.net/2433/122082
Right	
Type	Departmental Bulletin Paper
Textversion	publisher

HUMORAL CYTOTOXIC ANTIBODIES IN UROGENITAL CARCINOMA PATIENTS*

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ABSTRACT: A group of 124 patients with bladder, prostatic or kidney carcinoma, 18 patients with benign prostatic hypertrophy and 14 normal healthy controls were studied for the presence of humoral, complement-dependent, tumor-associated cytotoxic antibody. Human tumor tissue from kidney, prostate, and bladder was obtained from patients with a histologic diagnosis of adenocarcinoma or transitional cell carcinoma. Attempts to grow these tumors in culture resulted in no long-term cell lines but primary cultures were established so that they could be used as target cell cultures for the humoral cytotoxicity studies with patient sera. Serum cytotoxicity was demonstrated in both autochthonous and allogeneic experiments. Although autochthonous cytotoxic reactions were stronger, patient sera showing positive cytotoxicity in allogeneic experiments suggest cross-reacting, common antigens. Humoral cytotoxicity assays using long-term cell cultures indicate that the tumor-associated antigens detected on the surface of primary cell cultures were also present on long-term cell cultures.

MATERIALS AND METHODS

Cell Culture

Tissues, bathed in growth medium, were obtained directly from surgery and dissected into pieces 1–3 mm in diameter with a scalpel. Two methods were then employed for the *in vitro* growth of these small tissue pieces. 1) The immobilized explant procedure (Stone et al. 1975), consists of immobilizing the small tissue pieces beneath cover glasses in standard 60 mm petri dishes. Cover glasses are held firmly to the petri dish by a spot of sterile stopcock grease. Cell outgrowth is usually obtained within 3–5 weeks by this method. 2) The enzymatic dispersion technique (Stone et al. 1976) consists of incubating small pieces of

the tissue received from surgery overnight at 37° in 5% CO₂ with 5 ml of crude collagenase solution in a 60 mm Falcon petri dish. The tissue pieces are partially disrupted by the enzymatic activity of this crude collagenase. Gentle pipetting with a Pasteur pipet further disrupts the tissues such that the cells are dispersed as single cells and, if prostate is the tissue of origin, the prostate glands remain as large pieces. The cells are allowed to settle in a conical centrifuge tube for 15 min. before the liquid covering them is removed by gentle suction. This procedure results in the removal of the bulk of fibroblastic cells. Five ml of fresh growth medium is added to the settled cells as a wash and they are allowed to resettle for 15 min. The remaining cells are then pipetted into 60 mm petri plates and growth medium is added. The using cell culture lines confirmed which they have the tumor cells by the cloning method and the karyotype of chromosomes.

* Supported by Grant CA19840 from the National Prostatic Cancer Project, and Grants CA15292 and CA15972 from the National Bladder Cancer Project, National Cancer Institute, NIH, DHEW.

Reagents for tissue culture

Growth medium RPMI 1640 (Gibco) supplemented with 20% heat-inactivated fetal calf serum or bull serum, 0.3% tryptose phosphate broth (Difco), 0.0014% sodium bicarbonate, 0.005% streptomycin sulfate (Eli Lilly & Co.), 62.5 units/ml penicillin G (Squibb), 0.5 μ g/ml Fungizone (Squibb), 1 μ g/ml folic acid. Collagenase is Type 1, CLS (Worthington Biochemical Corp.), 1 mg/ml in growth medium filtered through 0.45 μ Nalgene filter.

Microcytotoxicity assay

The humoral cytotoxicity procedure as originally described by Takasugi and Klein (1970) was employed and modified as follows. Separate cultures of target cells grown *in vitro* were removed from the petri dishes with 0.25% trypsin. Supernatant containing trypsin, was removed by low speed centrifugation and target cells were resuspended in RPMI 1640 containing 20% fetal calf serum and adjusted to a concentration of 5000 cells per ml. Twenty μ l (approximately 100 cells), were delivered with an Eppendorf pipet to each of the 60 wells of Falcon 3034 microtest plates. The plates were allowed to incubate overnight to allow attachment of the target cells. The following day the medium was removed and 10 μ l of each test serum, previously treated at 56° for 30 min. to remove complements, were delivered to each of 6 wells. The plates were then returned to the incubator and allowed to incubate for 1 hr. Pooled guinea pig complement (Cappel Lab.) was used as a source of complements. This guinea pig serum was diluted in RPMI 1640 without fetal calf serum and was delivered in 10 μ l amounts to 3 of the 6 wells occupied by a given test serum. Guinea pig complements from the same pool in an identical dilution, but heated at 56° for 30 min. to inactivate the complement, was added to the remaining 3 wells occupied by a given test serum. Plates were then incubated for an additional 24 hrs. in the CO₂ incubator at 37°. Complements were then removed by flicking, the remaining adherent cells were washed with buffer to remove debris and stained with 0.3% trypan blue for 10 min. Plates

were again flicked and washed with buffer 3 times and then examined for evidence of cytotoxicity by viewing each well with a microscope and comparing the relative number of stained cells versus unstained adherent cells.

Sera

Blood samples were drawn from patients having prostatic carcinoma, benign prostatic hypertrophy or bladder carcinoma both before and after surgical removal of the tumor. Serum was separated, divided into small sample volumes, heated at 56° for 30 min. to remove complement, and stored in a -76°C Revco freezer. Sera from 162 individuals were assayed for complement-dependent cytotoxicity. Blood was collected from 14 normal healthy controls assured to be cancer free, 53 patients having bladder carcinoma, 30 patients having prostatic carcinoma, 41 patients having kidney carcinoma, and 18 patients having benign hypertrophy. In addition 6 patients having non-urogenital tumors were included.

RESULTS

Sera from the various patients were tested for cytotoxic activity against both short-term target cells and long-term tissue culture cell lines. Cytotoxic sera were found in all groups of patients and cytotoxicity was dependent on the exogenous guinea pig complement utilized. No toxicity occurred in any serum tested with guinea pig complement which had been heated at 56° for 30 min. The various tissue culture target cells utilized for

Table 1.

TISSUE CULTURE TARGET CELLS FOR MICROCYTOTOXICITY

Short-term target cells -

- 2 Bladder carcinoma
- 9 Prostate carcinoma
- 4 Kidney carcinoma
- 8 Benign prostatic hypertrophy
- 1 Normal prostate

Long-term cell lines

- T24 - (Bubenik et al, 1973) -- transitional cell carcinoma (bladder)
- 253J - (Elliott et al, 1974) -- transitional cell carcinoma (renal pelvis)
- 5873T - (Duke) -- renal cell carcinoma

Table 2.

HUMORAL CYTOTOXICITY AGAINST TISSUE TARGET CELLS

Diagnosis of serum donor	No. Sera Tested	Sera cytotoxic to No. (%)							
		Bladder Carcinoma	Prostate Carcinoma	Kidney Carcinoma	BPH	Normal Prostate	253J	T24	5873T
Bladder Carcinoma	53	13/25 (52)	63/447 (14)	17/141 (12)	29/221 (13)	8/80 (10)	65/143 (45)	80/153 (52)	4/68 (8)
Prostatic Carcinoma	30	0/18 (0)	178/466 (38)	10/138 (7)	91/220 (41)	16/36 (44)	0/46 (0)	11/36 (30)	0/27 (0)
Kidney Carcinoma	41	0/35 (0)	29/204 (14)	33/224 (15)	0/23 (0)	6/32 (19)	6/19 (32)	3/10 (30)	40/119 (34)
B.P.H.	18	N.T.	43/175 (25)	0/43 (0)	38/120 (32)	0/18 (0)	1/33 (3)	3/36 (8)	0/10 (0)
Normal	14	N.T.	20/45 (44)	0/34 (0)	3/35 (9)	1/9 (11)	1/13 (8)	1/15 (7)	0/6 (0)
Other	6	N.T.	10/58 (17)	0/18 (0)	2/33 (6)	2/6 (30)	N.T.	N.T.	N.T.

B.P.H. = Benign prostatic hypertrophy

N.T. = not tested

253J cells = transitional cell carcinoma - renal pelvis

T24 cells = transitional cell carcinoma - bladder

5873T cells = renal cell carcinoma

microcytotoxicity assays are shown in Table 1. Screening of the various sera against short-term target cells allows individual test serum samples to be exposed to various possible tumor-associated antigens. A total of 24 short-term target cells were utilized in the present study. The 3 long-term tissue culture cell lines were utilized so that comparisons could be made from the results of this study and the studies of others testing humoral cytotoxicity using similar cell lines. Line T24, a transitional cell carcinoma (Bubenik et al, 1973) and 253J, a transitional cell carcinoma (Elliott et al, 1973) and 5873T, originating in this laboratory from a renal cell carcinoma, were included. Rabbit heterologous antisera against these three individual cell lines were established such that positive cytotoxic controls could be included in all assays.

Table 2 shows the results of the humoral cytotoxicity activity against the various tissue target cells. The numbers in parentheses indicate the percent of cytotoxic sera found in each category. The fraction above the number in parentheses indicates the number of positive sera in each category over the total number of serum samples tested for that category. Serum was classified as positive for cytotoxic activity if it

killed 50% or more of the cells in each well. Each serum was tested in at least 6 wells and the results averaged.

It can be seen that bladder carcinoma serum was quite cytotoxic for bladder carcinoma target cells. In addition cytotoxic activity of bladder carcinoma patient sera was 45 and 52 percent respectively for 253J and T24 cells. Both of these are long-term cell lines which originated from transitional cell carcinomas. Therefore, bladder carcinoma patients' sera demonstrated both autochthonous and allogeneic cytotoxic activity. Bladder sera also detected what appears to be a transitional cell tumor-associated antigen. Testing of prostatic carcinoma patient sera demonstrated that 38% of the sera were cytotoxic to prostatic carcinoma target cells, 41% were cytotoxic to BPH target cells, and 44% were cytotoxic against normal prostate cells. This combination of cytotoxic activity may indicate the recognition by the test sera of common prostate-associated surface antigens. Kidney carcinoma test sera indicated a low percentage (15%) of positive cytotoxic reactions against kidney carcinoma test cells. This same group of kidney carcinoma test sera, however, was positive against each of the three long-

term cell lines tested. The 5873T cell line was a renal cell carcinoma-derived tumor and so therefore would be expected to be positive. The total number of assays on both 253J and T24 for kidney carcinoma test sera was low. Sera from patients having benign prostatic hypertrophy were found to be most cytotoxic when tested against short-term BPH test target cells. BPH patient sera as a group did not indicate the recognition of non-tumor prostate-associated antigens as did the prostate carcinoma sera. The 14 normal healthy control sera which were included in the test panel as negative controls were negative on all target cells tested with the exception of target cells from short-term cultures of prostatic carcinoma. Forty-four percent of normal sera tested against these prostatic carcinoma target cells were positive and the reason for this high percentage of cytotoxic activity is yet unexplained. The 6 patient sera from patients having non-urolological tumors which were included in our test panel, all had relatively low percent-positive reactions against test target cells.

DISCUSSION

A few general comments should be made concerning the microcytotoxicity test because two components, the tissue target cell culture should be selected from primary cultures of short-term growth since they would yield to the panel test sera surface antigens very closely related to what might be found in the patient. These tissue target cells had to be carefully selected and trypsinized from the 60 mm petri dishes prior to confluent growth, so that a short treatment with trypsin would yield single cells suspensions for subsequent plating in Falcon 3034 plates. Using cultures which have not grown to confluency also alleviated the problem of transferring clumps of cells into the microcytotoxicity plates. Antigens other than tumor-associated antigens may be present on tissue target cells. The tumor cell population *in vitro* may be less likely to grow than normal tissue under identical *in vitro* culture conditions. In many cases this has been apparent in these studies.

Therefore, normal, non-tumor cells may be more sensitive to complement-mediated damage than the tumor cells. In order to control for this variation in immunosensitivity of normal cells, the cytotoxicity caused by complement alone without the addition of test serum against both normal and tumor target cells from the same donor was utilized whenever possible.

In many microcytotoxicity assays using human target cells, the source of complement has been rabbit serum (Bloom, 1972; Hakala et al, 1974a, 1974b; Tamerius and Hellström, 1974; Canevari et al, 1975; Bodurtha et al, 1975). Rabbit serum, however, has a non-specific cytotoxic activity to many heterologous cells and usually pooled rabbit serum cannot be used without absorption because of this fact. Therefore, pooled guinea pig sera was used as the source of complement in these assays. Even so, it was important to control on each plate the percentage of cells surviving exposure to complement alone. In addition it was found that different pooled batches from the same source (Cappel Laboratories) had slightly different nonspecific cytotoxic activity to the cells and that the Cappel Laboratories source was by far the best source for low titers of nonspecific activity.

The antigens involved in positive cytotoxicity assays must be considered. Antigens other than tumor-associated antigens may also be involved in the *in vitro* detected immunoreactivity. Blood group substances may be expressed on the membrane of cultured cells (Bloom 1972). It is felt that blood group substances were not the main antigens detected in our assays since positive reactions were seen among serum donors matched for blood group with the donor of the target cells as well as those which were unmatched. Irie et al (1974) also suggested that heterologous membrane antigens are expressed on the surface of target cells. These antigenic expressions could have been detected and represented by the positive reactions in normal test sera against prostatic carcinoma cells but absorption studies could delineate tumor and histologic type-specific reactions. Additional studies are needed to find out whether the diffe-

rences noted in cytotoxicity levels and titers were due to different antigenicities or due to the difference in fragility of the test target cells. Additional absorption experiments are needed to define these specific reactions (Bloom, 1972). An additional variation, which may present in humoral assays, is that antigenic expression on the cell surface may be dependent on the growth cycle and other factors in the medium *in vitro* (Hakala et al, 1974a). For example, HL-A antigens may be expressed in greater concentration than tumor-associated or tumor-specific antigens and therefore be detected in humoral cytotoxic antibody assays. If absorption by autochthonous tumor can remove cytotoxicity, then the cytotoxic antigens expressed are not of the HL-A type but rather are directed against a neo- or tumor-associated antigen which is present *in vivo*. Nonspecific cytotoxic effects of serum were also important to control. Therefore, in each series of tests, the number of target cells surviving exposure to normal serum and complement was tested and compared to that surviving exposure to complement alone. With the exception of the cytotoxic effect of normal control sera on prostate carcinoma cells, normal serum had relatively little cytotoxic effect on the target cells utilized.

Correlation of clinical stage of disease with the reactivity of sera of urothelial tumor patients indicated that a patient's sera with positive cytotoxicity did not necessarily correlate with the patients' tumor burden. This is in contrast to findings with the patients' tumor burden. This is in contrast to findings of several other investigators looking at the same correlation (Lewis et al, 1969; Bodurtha et al, 1975; Canevari et al, 1975). They found that the degree of cytotoxic activity in patient sera varied inversely with the size of tumor burden, i.e. cytotoxic activity decreased as tumor burden increased. This concept, if applied to patients having bladder carcinomas, would predict that humoral cytotoxicity would be likely in patients having stage A and stage B carcinoma, decreased or absent in stage C and D, and perhaps increase after surgical

removal of the tumor burden. If a positive correlation had been found, it might have been clinically useful in monitoring the recurrence of tumor growth or metastatic spread in the patient.

Published evidence seems to favor the proposition that immune cells rather than humoral antibodies play the major role in graft rejection and also in the production of immunological activity directed against tumor cells *in vivo*. Hellström and Hellström (1974) state that the rejection of both syngeneic and autochthonous tumors are primarily by immune cells rather than humoral antibodies. The question then arises as to what extent tumor regression is due to a result of the destruction of tumor cells by complement-dependent, cytotoxic antibodies, and the humoral immune response. Is the measurement of humoral cytotoxic antibodies important? Is the measurement of humoral cytotoxic antibodies important? One should keep in mind that the test system utilized in these studies is an *in vitro* assay and that *in vivo* the humoral response may play quite an important role as tumor cell destroyers in the presence of complement. These humoral antibodies may also be a major factor in the tumor regression by acting as unblocking or arming antibodies, as suggested by the Hellströms.

In conclusion, these studies indicate that patients undergoing surgery for removal of urogenital tumors do exhibit low titers of humoral complement-mediated cytotoxic antibody directed towards tumor-associated antigens. Whether this cytotoxic antibody is playing a large role in the patients' immunological response to the tumor is not clear. The low antibody titers observed indicate that humoral antibodies probably play a minor role. At this time, the *in vitro* measurement of humoral cytotoxic antibody is not of direct benefit to the clinician in helping monitor the patient's immunological response to the tumor burden or in monitoring the patient's response to the clinician's post-surgical therapy.

ACKNOWLEDGEMENT

The authors would like to thank Dr. Kenneth R. Stone for growing, maintaining and identifying the target test cells used in these studies.

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(Accepted 1977. 3. 15.)

尿路癌患者における血清抗体に関する検討

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一般に癌は TSTA（腫瘍特異抗原）が証明され、腫瘍免疫の解析はリンパ球を中心にした細胞性免疫によってその研究がすすめられているのが現状である。

しかし、液性因子としての血清の問題はまだ完全に解決されていないし、その作用に関しては混沌としている。

ここに尿路癌の target cells に対し、その患者血清を用いて Takasugi-Klein の細胞障害性試験を補体依存性細胞性免疫能としての血清抗体の存在を検討した。

腫瘍組織は膀胱癌・前立腺癌・腎などの患者から外科的に採取し単層組織培養をおこない、それを攻撃

細胞として使用した、これらの培養細胞は cloning 法による pure culture および chromosome の検討により癌由来の細胞であることを確認したうえで使用した、検討血清は、患者血清（膀胱癌・腎癌・前立腺など）162 例と正常例14例を使用した。

この結果は50%以上の血清抗体の存在を認めた。これは尿路腫瘍における液性（血清）抗体活性はじゅうぶんに認められ、同時に tumor associated antigen と common antigen を認めたといえる。

今後、この面における研究を進め尿路癌の臨床に応用できるかどうか検討したいと思っている。